A complete pupillometry toolbox for real-time monitoring of locus coeruleus activity in rodents

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Abstract: The locus coeruleus (LC) is a region in the brainstem that produces noradrenaline and is involved in both normal and pathological brain function. Pupillometry, the measurement of pupil diameter, provides a powerful readout of LC activity in rodents, primates and humans. The protocol detailed here describes a miniaturized setup that can screen LC activity in rodents in real-time and can be established within 1–2 d. Using low-cost Raspberry Pi computers and cameras, the complete custom-built system costs only 300 euros, is compatible with stereotaxic surgery frames and seamlessly integrates into complex experimental setups. Tools for pupil tracking and a user-friendly Pupillometry App allow quantification, analysis and visualization of pupil size. Pupillometry can discriminate between different, physiologically relevant firing patterns of the LC and can accurately report LC activation as measured by noradrenaline turnover. Pupillometry provides a rapid, non-invasive readout that can be used to verify accurate placement of electrodes/fibers in vivo, thus allowing decisions about the inclusion/exclusion of individual animals before experiments begin.

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A complete pupillometry toolbox for real-time monitoring of locus coeruleus activity in rodents

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All authors have read and approved the final version of the manuscript.

EDITORIAL SUMMARY Pupil diameter is measured in rodents using low-cost cameras and a Raspberry Pi computer as a rapid, non-invasive real-time readout of locus coeruleus activity.

TWEET Miniaturized low-cost #pupillometry setup for measuring rodent pupil diameter as a real-time readout of #LocusCoeruleus activity. Featuring @DeepLabCut tracking

and an online toolbox for visualization and data analysis. @BohacekLab @MattiaPri @cbelephants

COVER TEASER Low-cost rodent pupillometry toolbox

RELATED LINKS

Key reference(s) using this protocol

Zerbi et al. Neuron 103, 702–718.e5 (2019) DOI: 10.1016/j.neuron.2019.05.034
Zuend et al. Nature Metabolism. In press (2020) DOI: 10.1038/s42255-020-0170-4

Abstract

The locus coeruleus (LC) is a region in the brainstem that produces noradrenaline and is involved in both normal and pathological brain function. Pupillometry, the measurement of pupil diameter, provides a powerful readout of LC activity in rodents, primates and humans. The protocol detailed here describes a miniaturized setup that can screen LC activity in rodents in real-time, and can be established within 1-2 days. Using low-cost Raspberry Pi computers and cameras, the complete custom-built system only costs around 300 euros, is compatible with stereotaxic surgery frames and seamlessly integrates into complex experimental setups. Tools for pupil tracking and a user-friendly Pupillometry App allow quantification, analysis and visualization of pupil size. Pupillometry can discriminate between different, physiologically relevant firing patterns of the LC, and can accurately report LC activation as measured by noradrenaline turnover. Pupillometry provides a rapid, non-invasive readout that can be used to verify accurate placement of electrodes/fibers *in vivo*, thus allowing decisions about the inclusion/exclusion of individual animals before experiments begin.

Introduction

The locus coeruleus – noradrenaline (LC-NA) system supports a vast number of fundamental brain functions. It modulates attention, arousal, synaptic plasticity and memory formation, it mediates the stress response, regulates sleep/wake states and is involved in cerebral blood flow regulation[1–3]. Dysfunction of the LC-NA system underlies numerous psychiatric pathologies including depression, anxiety disorders, stress susceptibility, PTSD and Down syndrome[4–7], and the loss of LC neurons is the earliest pronounced feature of both Parkinson's and Alzheimer's disease[8–12]. Despite its importance, the location of the LC deep in the brainstem, as well as its small size (~1500 neurons in each hemisphere in mice, ~20'000 in humans)[13,14] make systematic investigations of this nucleus technically challenging in both rodents and humans. Recent advances in optogenetics and pharmacogenetics enable precise labeling and manipulation of LC cells in rats and mice, yet proper placement of electrodes or optic fibers is still very challenging due to the size and location of the LC. Typically, researchers assess efficient targeting of the LC by *post mortem* histology/immunohistochemistry, leading to *post-hoc* exclusion of individual animals. While pupillometry – i.e. the measurement of changes in pupil diameter – is routinely applied for assessing LC function in humans and primates[15–20], its use in rodent models is not yet commonplace. Nonetheless, recent work shows that pupillometry provides an *in vivo* assessment of LC activity in awake or anesthetized rats and mice[21–25]. In this protocol we describe how to set up and use a pupillometry system, which we developed and previously used to assess modulation of the LC-NA system in awake as well as anesthetized mice[24,25].

Development of a miniaturized low-cost pupillometry setup. Commercially available setups (https://neuroptics.com/) to measure pupil diameter are not only costly but also bulky, preventing pupil measurements when space is limited, such as during stereotaxic surgery in rodents or in head-restrained two-photon calcium imaging setups. We established a custom-built pupillometry setup using ultra low-cost Raspberry Pi computers and cameras[24,25]. This customizable system requires minimal space and seamlessly integrates even into narrow experimental setups (**Fig. 1a-c**). We use MATLAB-based data analysis to facilitate a fast and simple measurement of pupil radius over time. In our experience, this is sufficient to validate accurate stimulation of the LC. However, reliable and high-quality pupil tracking requires successful handling of various experimental constraints, including poor contrast under low-light conditions, spontaneous pupil movement and interference from reflections. Therefore, as an alternative to the MATLAB-based analysis, we have also used the recently described motion tracking software DeepLabCut[26,27] for precise, robust and versatile pupil tracking. We developed a user-friendly, web-based *Pupillometry App* to facilitate data visualization and analysis. Here, we provide a detailed workflow that describes every step from building the setup to data collection, data processing and data analysis (see **Fig. 1d**). All of the required software is freely available online, allowing the tracking, analysis and quantification of pupil size to be performed using any lab computer. We also provide protocols describing how to perform pupillometry recordings in darkness (**Box 1**), to measure pupil changes in response to light stimuli (**Box 2**), and to quantify relative as well as absolute pupil size (**Box 3**). In the 'Anticipated Results section' we demonstrate that pupillometry is sensitive enough to discriminate between different physiologically relevant firing patterns of the LC, and that it can accurately report LC activation as measured by noradrenaline turnover. Pupillometry is thus a rapid, non-invasive tool to verify accurate

placement of electrodes/fibers *in vivo*, allowing decisions about the inclusion/exclusion of individual animals at an early stage of ongoing experiments.

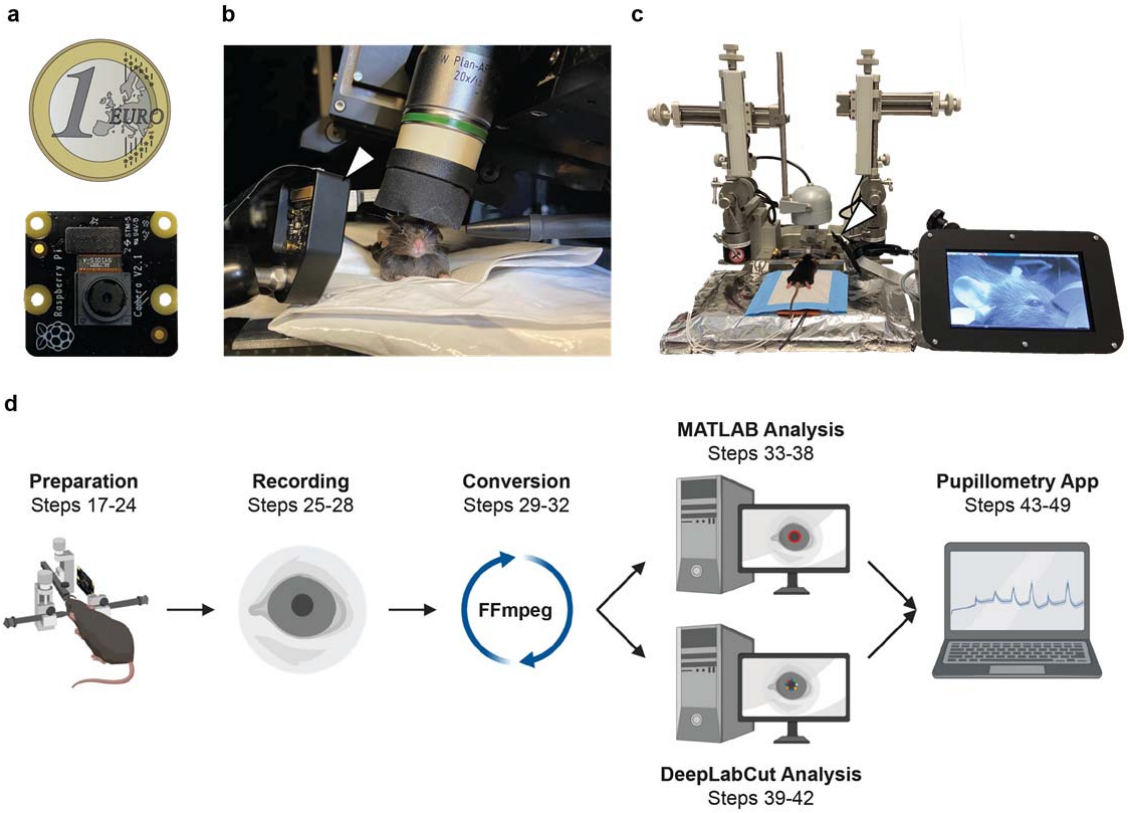


Fig. 1: Pupillometry using a small and light-weight camera. (a) The Raspberry Pi NoIR Camera V2 Module is approximately the same size as a 1-Euro coin. **(b)** The camera module (inside custom housing and indicated with white arrow tip) in a two-photon microscopy setup. **(c)** Camera module (indicated with white arrow tip) in a stereotaxic surgery setup connected to a Raspberry Pi with touch screen. An additional infrared flood-light illuminates the mouse from above. **(d)** Workflow overview for pupillometry recordings and data analysis. Steps from the written protocol are indicated for each section. Created with Biorender.com.

Advantages and limitations of the technique. Apart from pupillometry, no currently available technique provides a non-invasive real-time quantification of LC activity *in vivo*. However one limitation of the current technique is that pupil diameter changes are part of the response of the visual system. There is no correlation between luminance-mediated changes in pupil diameter and LC-mediated changes, thus it is necessary to correct for changes in luminance when performing experiments (see **Box 2**). In addition, the LC has a modular organization, where certain ensembles of cells project to specific brain regions[28,29]. As a consequence, only those LC neurons that project to the Edinger-Westphal nucleus and/or the superior cervical ganglion can elicit a pupil response via parasympathetic and sympathetic pathways[30]. While stimulation of the entire LC triggers a robust pupil response, retrograde labelling techniques can be used to manipulate specific ensembles of LC neurons. Using this technique, we have observed that selective stimulation of LC neurons that project to the hippocampus do not result in pupil dilation (unpublished observation).

Therefore, pupillometry may not always be a suitable readout of projection-specific LC activity.

The only alternative real-time readouts of LC and noradrenergic activity use invasive recording or imaging techniques, the latter involving novel, genetically encoded noradrenaline sensors[31–33]. These techniques require extensive technical and experimental expertise and may not be available to the majority of labs conducting research on the LC-NA system. While pupillometry has long been a widely used clinical measure, the advent of fMRI-compatible eye-tracking systems has popularized its use in human neuro-imaging research[15,34]. While pupil dilation during brain imaging has often been considered a nuisance variable that needed to be controlled for statistically[35,36], it has recently emerged as a relevant biological variable in the context of arousal and noradrenergic activation, and is now a widely-used measure in human cognitive neuroscience[20,34–36]. Popularizing pupillometry in rodent models - where the LC-NE system can be systematically manipulated - will help elucidate the neuronal processes and brain-states that drive the pupil response, and reveal how they relate to behavior in health and disease[20,21,24,37]. Pupillometry can thus provide an important translational link for comparative studies using both preclinical and clinical data.

The miniaturized low-cost setup in combination with DeepLabCut-based tracking is versatile and can be used for any tracking task such as tracking the movement of whole animals or body parts (e.g. limbs, fingers, whiskers). Therefore, our protocol describes a low-cost, miniaturized video tracking device compatible with any task that requires video recording and tracking over time. A very promising recent approach was the development of a head-mounted light-weight camera device, allowing pupil tracking in freely moving rodents[37]. Our setup uses the same camera system and our analysis pipeline is compatible with the head-mounted system, although this approach is not described here.

Experimental Design

Animals and experimental parameters. This protocol has been optimized for validating LC activation in C57Bl6/J mice, but in principle it can be used to measure changes in pupil size in various setups and species, as long as the pupil is clearly visible with sufficient contrast between pupil and iris. Due to these constraints, pupil tracking may be challenging in albino strains or in animals that suffer from pathological eye conditions (e.g. cataracts). Using light stimuli to trigger changes in pupil diameter is an efficient positive control procedure to ensure successful recording of changes in pupil size (see Box 1). We have not detected any sex-dependent differences in the pupil response when manipulating LC function in mice (**Fig. 4b**). For experiments in our lab, we use young adult mice (aged 2-4 months), however, we expect pupillometry to work equally well in younger or older animals. While pupillometry provides an easy and fast readout of LC activation when performed in anaesthetized animals[24], we have previously shown that our setup can also be used to record from awake animals[25]. This however requires experimental animals to be head-restraint and trained accordingly, which is technically more difficult and time consuming.

Locus coeruleus manipulations and pupillometry. Using pupillometry recordings to evaluate manipulation of the LC requires stereotaxic surgeries. Surgical procedures must be performed according to the regulations and guidelines of the local authorities, and animals must be allowed enough time for recovery. When viral vectors are delivered, proper virus

expression is necessary, and pupillometry can be used to assess whether virus expression is sufficient to allow LC activation in a given animal (see **Fig. 4c,d**). For electrical and opotogenetic stimulation of the LC, many labs use unilateral activation, in which case pupillometry recordings should be performed on the ipsilateral eye, where LC activation elicits a larger pupil response[30]. Because the pupil is sensitive to light, special precautions should be taken when using optogenetic LC activation during pupillometry recordings. Excessive light spreading from the optic fiber should be avoided (e.g. use dark nail polish to paint dental cement). That being said, we have controlled for this possibility during by including a "laser-only" control group, and we have not detected changes in pupil size due to the 473nm laser light itself (**Fig. 3b**). However, this might be different for other stimulation parameters and laser wavelengths, thus the inclusion of proper control groups is important.

Baseline and Power. While we show that a broad range of LC stimulation protocols can be used to manipulate pupil size, it is critical to include an appropriate baseline recording prior to LC stimulation. A stable baseline recording avoids artifacts from unspecific fluctuations in pupil size, and also enables proper statistical analyses. Importantly, our protocol is intended to test the efficacy of LC stimulation within a single animal, thus analysis of pupil size over time can directly reveal the effect of LC stimulation, as shown by recordings for individual mice in Supplementary Figure 2. In some cases, changes in pupil size need to be compared between experimental groups, for example to test the effect of different LC stimulation paradigms. We performed a statistical power analysis based on data from optogenetic LC activation (5 Hz/ 10 mW for 10s; Fig. 3b, n=9/10), which revealed that no more than 3 mice per group are necessary to find a significant effect of optogenetic stimulation between ChR2-EYFP animals compared to EYFP controls in 95% of experiments (alpha < 0.05 and beta < 0.05).

Materials

Biological materials

- For all experiments shown, we have used adult mice from either C57BL/6J or C57BL/6-Tg(Dbh-icre)1Gsc (DBH-iCre)[38] mouse strains.

  CRITICAL

  In order to manipulate locus coeruleus activity, prior surgical interventions are necessary to i) implant stimulation electrodes (e-stim), ii) inject viral constructs (chemogenetics and optogenetics), and/or iii) implant optic fibers (optogenetics). The procedures we have used are described in Supplementary Methods.

  CAUTION

  All experiments must be done in accordance with guidelines and regulations of the relevant authorities. These procedures were approved under licenses ZH161/17 and ZH169/17 by the local veterinary authorities, conforming to the guidelines of the Swiss Animal Protection Law, Veterinary Office, Canton Zurich (Act of Animal Protection 16 December 2005, and Animal Protection Ordinance 23 April 2008).

CAUTION

In principle, this pupillometry protocol should be compatible with tracking the pupil in most vertebrate species that have normal eye pigmentation. The setup has been optimized for C57Bl/6 mice, where tracking is rather challenging due to poor contrast between iris (brown) and pupil (black). However, detecting and tracking the pupil in albino animals is difficult and might require special adjustments to achieve the necessary contrast between pupil and iris.

Reagents

- Isoflurane

CAUTION

We do not recommend the use of injectable anesthesia, as many commonly used drugs (e.g. medetomidine and xylazine) impact noradrenergic activity.

Equipment

Software

- Raspbian OS
  (https://www.raspberrypi.org/downloads/)

- balenaEtcher
  (https://www.balena.io/etcher/)

- Raspberry Pi Code
  (https://github.com/ein-lab/pupillometry-raspi)

- FFmpeg
  (https://ffmpeg.org/)

- MATLAB
  (https://www.mathworks.com)

- MATLAB Code
  (https://github.com/ein-lab/pupillometry-matlab)

- DeepLabCut
  (http://www.mousemotorlab.org/deeplabcut)
  (https://github.com/AlexEMG/DeepLabCut)
  See Nath et al 2019[27]

- *Pupillometry App*
  (https://bohaceklab.hest.ethz.ch/pupillometry/ (web version) or
  https://github.com/ETHZ-INS/pupillometry (source code))

Hardware

- Computer

  CAUTION

  Special computer specifications are required for the use of DeepLabCut. See Nath et al. (2019)[27] for more details.

- Micro SD Card and adapter (https://www.transcend-info.com/Embedded/Products/No-423)

- Raspberry Pi 3 B/B+ (or newer) (https://www.raspberrypi.org/products/)

- Raspberry Pi Touchscreen Display (https://www.raspberrypi.org/products/raspberry-pi-touch-display/)

- Raspberry Pi NoIR Camera V2 (https://www.raspberrypi.org/products/pi-noir-camera-v2/)

- CSI Flex cable (1 meter) (https://www.adafruit.com/product/2143)

- 2x power supply cables for Raspberry Pi and touch screen (https://www.raspberrypi.org/products/raspberry-pi-universal-power-supply/)

- Infrared light source (https://www.amazon.com/Infrared-Illuminator-Power-Vision-Camera/dp/B01D73XM24/)

  CAUTION

  The size of the infrared light source should be chosen to fit the given setup.

- Mass storage device (USB flash drive or harddisk)

- Wireless keyboard and mouse

- Screwdriver

- Suitable holding mechanisms/arms for camera and infrared light source (For example: https://www.amazon.com/Toolour-Soldering-Rotatable-Alligator-Electronics/dp/B07JVF88TV/)

  CAUTION

  Holding mechanisms/arms should be chosen to fit the setup, ideally allowing for flexibility whilst providing stability for the camera and infrared light.

- Luxmeter

- Stereotaxic frame & anesthesia set-up

Procedure:

Pupillometry equipment setup (Timing 2 hours)

CAUTION

Further information on how to build this pupillometry setup can be found on: https://ein-lab.github.io/pupillometry-raspi/

1. Download the Raspbian operating system and write it to an SD card using balenaEtcher.

CAUTION

Raspbian is available in 3 different bundles. Download “Raspbian with desktop and recommended Software”. For a detailed description on how to install the Raspbian OS visit:https://www.raspberrypi.org/documentation/installation/installing-images/

TROUBLESHOOTING

2. Before assembly, make sure all hardware and tools are ready and software preparation is completed.

3. Place the Raspberry on the brass spacers of the touch screen board. Make sure the micro-USB connectors on both boards are aligned and fasten the Raspberry in place with the four Phillips screws provided.

4. Install the SD card prepared in step 1.

CAUTION

The pins must face the Raspberry’s PCB. Care must be taken in order to avoid damage to the SD card slot.

5. Connect the screen PCB and the Raspberry Pi using the short ribbon cable that came with the touch screen.

CAUTION

Make sure the metal contacts on the ribbon cable are facing towards the contacts inside the socket, and away from the black bracket.

CAUTION

Ignore the 4 colored cables provided with the touch screen. These are only needed if you use an older Raspberry Pi and/or if you plan to power the Raspberry from the screen directly. This option is not recommended, as screen, camera and light need more power than a single average power supply can provide.

6. Connect the long CSI flex cable for the camera to the remaining socket on the Raspberry Pi. Lift the black bracket and push the cable in, then lock it in place by replacing the bracket.

   CAUTION
   Make sure the metal contacts on the ribbon cable are facing towards the contacts in the socket, and away from the black bracket.

   CAUTION
   Please be aware that this part of the assembly will likely not be accessible anymore once it is inside the housing.

7. Install the housing and secure it with the provided screws. Ensure correct orientation of assembly.

8. Connect the Raspberry NoIR V2 camera to the far end of the long ribbon cable.

   CAUTION
   Make sure the metal contacts on the ribbon cable are facing towards the contacts in the socket, and away from the black bracket.

9. (Optional) Connect mouse and keyboard to the Raspberry Pi's USB ports. Although a computer mouse is not necessary when using a touch screen, it simplifies handling of the system.

10. Power the system by connecting the first micro-USB power supply with the socket on the touch screen board (the one closer to the screen) and then the second power supply to the second micro-USB socket.

    CAUTION
    Always power the touch screen first; otherwise the Raspberry Pi will start without display output.

    CAUTION
    Depending on your display stand or case, you might find the screen is upside-down. This setting can be changed. Please refer to the Troubleshooting section.

    TROUBLESHOOTING

11. Wait for the Desktop screen to appear and open a terminal window from the menu bar.

12. Test if the camera is detected by running the following command and press "Enter". The output should state *supported=1 detected=1*.

    ```
    vcgencmd get_camera
    ```

TROUBLESHOOTING

PAUSE POINT
The protocol can be paused after step 12 and continued at a later time point.

Software setup (Timing 1 hour)

13. ***Raspberry Pi.*** Connect the Raspberry Pi to the internet, either via WiFi or ethernet.

14. Open a terminal window from the menu bar.

15. Enter the following command and press “Enter”.

```
curl -sSL https://git.io/JeKP0 | bash
```

CAUTION
This will run a series of commands to install necessary software and enable the relevant Raspberry Pi settings. The Raspberry Pi will reboot after completion.

16. ***Computer.*** Download and install either FFmpeg with MATLAB and our MATLAB analysis files (https://github.com/ein-lab/pupillometry-matlab) or FFmpeg with DeepLabCut (https://github.com/AlexEMG/DeepLabCut).

CAUTION
Help with DeepLabCut installation and setup can also be found here:
http://www.mousemotorlab.org/deeplabcut

PAUSE POINT
Steps 13-16 only have to be carried out once. After the hardware and software have been set up, the protocol can be paused and continued at any later time point.

Experiment Preparation (Timing 10 min/animal)

17. Before starting the experiment, ensure that equipment, tools and reagents are ready.

18. Set your experimental room illumination.

CAUTION
Illumination intensity can be adapted to match experimental settings, but must remain constant for any given experiment, as lighting intensity will affect pupil size. For all experiments shown in the “Anticipated Results” section, room illumination was measured within the stereotaxic frame and set to 25-35 lux. Recordings in darkness require some adjustments, which are described in **Box 1**.

19. Connect the memory device (USB flash drive or external hard disk), wireless USB keyboard and mouse to the USB ports of the Raspberry Pi. Turn on the Raspberry Pi and display by connecting them both to a power supply.

    CAUTION
    Always power the display before powering the Raspberry Pi. Otherwise, the screen will remain black.

    TROUBLESHOOTING

20. Once the Raspberry Pi has launched, open the terminal and enter the following command to set a save location on your memory device. Confirm by pressing “Enter”.

    ```
    cd /media/pi/your_memory_device/experiment_folder
    ```

    CAUTION
    The Raspberry Pi itself possesses only limited memory capacity, therefore the use of an external memory storage device is highly recommended.

    TROUBLESHOOTING

21. Start a grayscale video stream by using the “raspivid” command in the terminal as in the following example and press “Enter”. A live video stream from the camera will appear on the screen.

    ```
    raspivid -t 0 -rot 180 -sat -100
    ```

    CAUTION
    The `-rot n` command rotates the video output by “n” degrees. Dependent on your setup, the rotation command may not be required.

    TROUBLESHOOTING

22. Induce anesthesia by exposing the mouse to 4% isoflurane. Once the animal is fully asleep, place the animal into the stereotaxic frame under 2% isoflurane (constant flow).

    CAUTION
    Keep animals in the stereotaxic frame on a heating pad (at body temperature) to guarantee the well-being of experimental animals, especially during long recording sessions.

23. Prepare the animal for LC stimulation. Depending on the chosen LC manipulation strategy, attach the optic fiber patch cord, implant and/or connect the electrode or set an intraperitoneal catheter for drug administration. Additionally, lightly fix the earbars so that the animal is stable in the stereotaxic frame.

CAUTION

Noxious stimuli elicit pupillary responses, handling the animals in the stereotaxic frame should be done carefully to avoid unnecessary pupil dilation.

CAUTION

Light head fixation reduces movement artifacts that may cause problems during pupil tracking.

24. Position the infrared light source and the camera around the animal so that the pupil is clearly visible, illuminated and in focus. Avoid reflections that overlap with the pupil if possible. In case the pupil is fluctuating in size and/or moving, wait until the pupil has stabilized and settled in the visible part of the eye before starting to record.

CAUTION

The quality of recorded videos determines how well the pupil tracking will work. Aim for the best contrast, avoid infrared reflections within the pupil, and animals with already widely dilated pupils. The MATLAB algorithm is very sensitive to artefacts. Reflections on the pupil (especially on the pupil border) may lead to tracking problems.

TROUBLESHOOTING

Pupillometry recording (Timing 5-30 min/animal)

CRITICAL

This protocol has been optimized for short (<20 min) pupil recordings. To assure the well-being of experimental animals, we advise to keep recordings as short as possible (<20 min), or use of artificial tears (SYSTANE Hydration; 1 drop every 30 min) to avoid damage to the eye. The use of artificial tears might affect pupil tracking.

25. End the video live stream with the key combination “Ctrl + C” on the Raspberry Pi.

26. Start recording a video with the Raspberry Pi camera, by using the `raspivid` command in the terminal with slightly modified parameters, as seen in the following example code, and press “Enter”. This will immediately start recording a video, according to the set video parameters in the command, and save it under the selected name. This command has to be adjusted to match the individual experimental settings. The example here starts a grayscale video (Videoname.h264) for 10 min at 5 frames per second with the contrast set to 40.

```
raspivid -o Videoname.h264 -sa -100 -vs -rot 180 -fps 5 -t
6000000 -co 40
```

CAUTION

We advise testing and choosing video parameters for a given experimental setup before starting experiments. For a more detailed description of the `raspivid` command and how to personalize video recording parameters see: https://www.raspberrypi.org/documentation/raspbian/applications/camera.md

CAUTION

Coordinate this step with the chosen LC manipulation. It is advised to include a baseline recording of at least 1-2 min before starting the stimulation.

CAUTION

The analysis speed in later steps (using MATLAB or DeepLabCut analysis) depends on the length of the video and the number of frames.

TROUBLESHOOTING

27. The recording willstop automatically if the timeout parameter `-t` was used in step 26. The recording can also be or can be terminated manually by using the key combination “Ctrl + C”. The video file will be saved to the specified location as an “.h264” file.

28. Once the recording is finished, remove the animal from the stereotaxic frame and let it recover from anesthesia. To continue with pupillometry recordings repeat steps 21-27 or alternatively turn off the Raspberry Pi by issuing the command `sudo halt` in the terminal and press “Enter”.

CAUTION

After executing the command `sudo halt` the display goes black immediately, even though the Raspberry Pi is still shutting down. Allow at least 30 seconds before unplugging the device from the power supply.

CAUTION

Use unique names for each recording. Otherwise, previously recorded videos will be overwritten.

PAUSE POINT

All downstream steps can be carried out at a later time point.

Conversion of video files to “.mp4” with FFmpeg (Timing 2-3 min/video)

29. Connect your memory device to the computer where FFmpeg and MATLAB or DeepLabCut was installed.

30. Open “Command Prompt” terminal on the computer and use the `cd` command to set the working directory as the location of the “.h264” video files and press “Enter”.

```
cd path_of_the_video_folder
```

31. With FFmpeg convert each ".h264" video file to ".mp4" format. Simultaneously, videos can be cropped to show only the eye which will facilitate further video analysis. For example:

```
ffmpeg -framerate 5 -i Videoname.h264 -filter:v
"crop=1000:1000:1000:1000" Videoname.mp4
```

CAUTION
It is important to specify the same frame rate that was used in step 26 when recording the video. Cropping videos down to the size of the eye is recommended to facilitate efficient video analysis. The size and location of the cropping window may have to be adapted between videos.

CAUTION
For a more detailed description on how to use FFmpeg and add other features please visit: https://ffmpeg.org/

TROUBLESHOOTING

PAUSE POINT
All downstream steps can be carried out at a later time point.

Analysis of video files

32. Proceed with analyzing the MP4 video files using option A for pupil tracking with MATLAB or option B for pupil tracking with DeepLabCut.

A) Analysis of video files with MATLAB (Timing 2-5 min/video)

i) Open MATLAB and in the file browser navigate to the pupillometry-matlab folder (see step 16).

ii) Right-click the folder and select "Add to path" -> "Selected Folders and Subfolders".

iii) In the command window, type `pup01 = pupilMeasurement()` and press "Enter".

CAUTION
This runs `pupilMeasurement()` without any additional parameters. Optional parameters can be adjusted and are given as name-value pairs, for example `pupilMeasurement('doPlot', true, 'frameInterval', 5)` to display a plot and analyze only every fifth frame. For a list of all available optional parameters, please refer to the MATLAB sample script from the GitHub repository, or type `help pupilMeasurement`.

TROUBLESHOOTING

iv) From the dialog box, choose one or several MP4 video files and click “Open”. Then select a directory to save the output.

v) MATLAB now presents you with the first frame of the first video file. Indicate the diameter across the pupil by left-clicking the left edge of the pupil and right-clicking the right edge of the pupil.

CAUTION
Try to draw the longest diameter across the pupil in order to provide as many seed points as possible. Right-clicking confirms the selection and the window closes immediately.

CAUTION
The experimenter should be blinded for group assignment to avoid any labelling bias.

vi) Wait for the progress bar to reach 100% and repeat step iv for all videos. Results will now be saved as “.mat” files in the results folder indicated in step iv.

TROUBLESHOOTING

PAUSE POINT
The results can be analyzed in the *Pupillometry App* (step 33) at a later time point.

B) Analysis of video files with DeepLabCut (Timing 5-10 min/video)

CRITICAL
Pupil tracking with DeepLabCut (DLC) requires a network that has been trained on a set of videos to recognize the pupil. The DLC network that was used for pupil tracking in this protocol is freely accessible on https://zenodo.org/deposit/3631569, DOI:10.5281/zenodo.3631569. Although we provide our own DeepLabCut network, we strongly recommend training a new network tailored to each individual setup. New DeepLabCut networks can be based on our pre-trained network to facilitate the training process. A detailed description for training DeepLabCut networks has recently been provided by Nath and colleagues[27].

i) Open Anaconda and start an IPython session within your DeepLabCut environment and import the package with the following command and press “Enter”:

```
import deeplabcut
```

ii) Set the config path according to your config file location and press “Enter”.

```
config_path = 'path_of_the_config_file'
```

iii) To analyze videos use the following command and indicate the path to the MP4 video files. To start the analysis press “Enter”. Analysis data will be saved in the MP4 video file folder as “.csv” files.

```
deeplabcut.analyze_videos(config_path,
['path_of_video_to_be_analyzed'], save_as_csv=True)
```

iv) Once all videos have been analyzed, labelled videos can be created for a visual assessment of performance using the following command and press “Enter”. This will generate the labelled videos and save them into the folder of the original MP4 videos.

```
deeplabcut.create_labeled_video(config_path,
['path_of_previously_analyzed_video'])
```

CAUTION

It is good practice to check each labelled video to confirm that pupil tracking was successful.

TROUBLESHOOTING

PAUSE POINT

The results can be analyzed in the *Pupillometry App* (step 33) at a later time point.

Visualization and statistical analysis with the *Pupillometry App* (Timing 10-20 min)

33. Access the *Pupillometry App* from your internet browser via https://bohaceklab.hest.ethz.ch/pupillometry/ , or install the package locally from the source at https://github.com/ETHZ-INS/pupillometry .

    CAUTION
    The *Pupillometry App* provides a detailed user manual (see **Supplementary Manual**) and an example dataset to guide new users through the app.

34. Navigate to the "Files & samples" tab and directly upload your ".mat" data files (MATLAB analysis) or for the DeepLabCut pathway upload the ".csv" data files into in the "Upload and transform DLC files", which will automatically read and transform them. In addition, upload a ".csv" metadata file with the necessary experimental parameters (Animal ID, Group, etc.).

    CAUTION
    The "Upload and transform DLC files" tab also allows you to download a metadata file template that already contains the filenames of the uploaded files. This can also be used with ".mat" files.

    CAUTION
    If videos have varying frame numbers, use the "Align files" button to equalize the number of frames.

    TROUBLESHOOTING

35. Go to the "Bins" tab, indicate the used frame rate and set baseline and stimulation bins according to the experimental settings.

36. Check each animal trace and if necessary remove tracking artefacts from your data in the "Clean up" tab.

37. Customise Pupillometry plots in the "Settings" tab if required.

    CAUTION
    Grouping variables have to be set in the ".csv" metadata file.

    TROUBLESHOOTING

38. Go to the "Plot" tab to view and export pupillometry plots.

39. If needed, statistical analysis of pupillometry experiments can be performed in the "Statistical tests" tab or raw data can be exported via the "Export" tab for external analysis.

Troubleshooting:

See Table 1 for troubleshooting guidance.

Table 1: Troubleshooting

Step	Problem	Possible reason	Solution
1	MicroSD card not writable	SD card adapter's write protection switch is in wrong position	Move the slider on the SD card adapter
10	Screen remains black	Components powered in wrong order	Power the screen first and the Raspberry Pi second
		Cables not properly seated	With the Raspberry Pi turned off and power disconnected, reseat all ribbon cables and carefully check for correct orientation
10	Display upside down	Screen setting needs to be adjusted	Edit the file /boot/config.txt on the microSD card and add a new line `lcd_rotate=2` to the bottom of the file
12/21/26	Camera not detected	Cable not properly connected	With the Raspberry Pi turned off and power disconnected, remove camera ribbon cable, check the orientation of the pins towards the pins in the socket and reconnect
19	Raspberry Pi hangs on boot	An unexpected power cut might have corrupted data on the microSD card	Remove the microSD card and repeat steps 3-5 and 9
19	Screen shows yellow lightning symbol	Raspberry Pi's power supply is undersized	Use a USB power supply that supplies at least 2.5 A
20	External hard disk not recognized	The Raspberry Pi may not supply enough power to the drive	Try using a powered USB hub to connect the drive or use a hard drive with external power
24	Video is too dark and the pupil is barely visible	Infrared light is turned off or too far away from the animal, which leads to a bad contrast	Check the infrared light and move it closer to the eye
24	The pupil is visible but is obscured by reflections	Angle of the infrared light to the pupil is not optimal	Move the infrared light around to find a good spot where the eye is well illuminated and the reflections are on the outskirts of the eye, where they are unlikely to interfere with pupil tracking
24	Pupil of the animal is extremely dilated from the start	This can either be related to lighting conditions (darkness), pain, or it can occur without any obvious reason	Check the animal for possible pain sources like too tight ear bars and relieve the pressure. If there are no obvious noxious stimuli, wait a few minutes to see if the pupil

			constricts by itself. In case this does not help, short light exposure (1-2 s) induces a strong pupil constriction. Over the next few seconds, the pupil slowly adapts to the ambient lighting conditions, and the recording can begin once the pupil size has stabilized. If this does not solve the problem either, continue with the next animal and try again later.
24	Pupil of the animal is moving around and/or moving outside the visible part of the eye	This usually happens within the first few minutes of the animal being in the setup	Wait until the pupil has settled by itself in the visible part of the eye before starting to record
24	A milky white spot covers the eye of the animal which is not related to light reflection	The animal suffers from retinal damage and cataract formation	Try recording from the other eye. If both eyes are affected the animal has to be excluded
31	Converted videos are the wrong length	The frame rate of the recording and the conversion do not match	Make sure you specify the same frame rate both when starting a recording and when converting the videos
31	Cropped video does not include the whole eye	Cropping parameters were wrong for the given video	Change the cropping parameters and convert again
32A iii	MATLAB Error: Undefined function or variable ‘pupilMeasurement’	Toolbox folder not on MATLAB path	Right-click the folder containing contents from pupillometry-matlab repository and select "Add to path" and "Selected folders and subfolders"
32A vi	MATLAB Error: No circular structure for radius r in this frame	MATLAB is unable to identify pupil	Re-run and carefully select line at a different angle
			Re-run with `'enhanceContrast'` flag enabled and/or set `'startFrame'` to a different value
			Inspect the video for resolution, pupil contrast and artifacts during the whole recording
32B iv	DeepLabCut did not track the pupil properly	This could be a video or network issue	If the video is too dark, try to change its brightness/contrast and analyze again. If it is not video related, train a new DeepLabCut network based on diverse videos to improve the flexibility of the network.
34	“Files & samples” tab shows “error” after uploading data and metadata files	There is a mismatch between data files and metadata information	Check the metadata file and correct wrong file names/spelling mistakes. Do not use any special characters in filenames or variables (+*#&;’%? etc.).
34	Missing files after DLC import	Computation of the pupil diameter was not possible for some files due to bad tracking	Optimize DLC tracking or reduce likelihood cutoff and/or completeness cutoff to ensure that

		quality	the center point and at least one pupil edge point are retained
37	“Plot” tab shows no graph after setting plot parameters	There is a problem either with other settings within the app or with the grouping variables in the metadata file	Check all settings. For example a normalized graph requires a baseline bin and will not work unless a baseline bin is set.
37	Ribbon plots are broken up / show artefacts	There is a problem with missing data in plotting intervals	Increase the interval size in the “Settings tab”
39	No statistical results are shown	There is a problem with either the bins or any variable	Ensure proper bins are defined in the “Bins” tab. In the “Statistical tests” tab, ensure the testing variable and animal variable are correctly set. If you use your custom model ensure a proper null model is provided. Ensure variables allow for the selected test (e.g. a variable with only one group cannot be used to test group differences).

Timing

Steps 1 - 12, Equipment setup: 2h

Steps 13 - 16, Software setup: 1h

Steps 17 - 24, Preparation: 10 min/animal

Steps 25 - 28, Pupillometry recording: 5 - 30 min/animal

Steps 29 - 31, Convert video files to “.mp4” with FFmpeg: 2 - 3 min/video

Steps 32A, Analyze video files with MATLAB: 2 - 5 min/video

Steps 32B, Analyze video files with DeepLabCut: 5 - 10 min/video

Steps 33 - 39, Visualization and statistical analysis with the *Pupillometry App*: 10 - 20 min

Anticipated Results

Pupillometry as a real-time readout of LC activation.

The relationship between LC activity and pupil diameter is well established[23,30,39]. Here, we take advantage of this tight relationship to demonstrate that pupillometry provides a sensitive real-time readout of *in vivo* LC manipulation. To strongly and selectively activate the LC, we first used Designer Receptors Selectively Activated by Designer Drugs (DREADDs, hM3Dq), which can stimulate LC firing[40], trigger NA release throughout the brain, and elicit a strong and long-lasting pupil dilation[24,41]. Transgenic mice that include codon-improved Cre recombinase (iCre) under the dopamine-beta-hydroxylase (DBH) promoter (DBH-iCre mice) received stereotaxic injections of a virus expressing either excitatory DREADDs (AAV5-hSyn-DIOhM3Dq-mCherry; hM3Dq-mCherry) or mCherry alone (AAV5-hSynDIO -mCherry; mCherry) bilaterally into the LC (coordinates from bregma: +5.4 mm AP; ±1.0 mm ML; -3.8 mm DV) (**Fig. 2a**). Pupil recordings were performed unilaterally. Baseline pupil size was recorded for 2 min before administration of an ultra-low dose of the potent DREADD agonist clozapine (0.03 mg/kg intraperitoneally [i.p.])[24,41]. Within a minute of clozapine injection, we observed a slow but steady pupil dilation in the hM3Dq-mCh group, which led to a strong and long-lasting increase in pupil dilation throughout the recording period **(Fig. 2b)**. In contrast, the pupil size of control mice (mCherry) did not change in response to clozapine injection and remained stable throughout the 10 min recording session. This shows that a short pupillometry recording reliably reports chemogenetic LC activation in real-time. All tracking in this report was performed with DeepLabCut and all analyses use linear mixed effects models[42], an analysis comparable to repeated one- or two-way ANOVAs. This allows us to correct for changes in pupil radius that are dependent on interindividual differences between animals and on baseline drifts over time. All effect sizes/standard errors are reported in pixels. For detailed description and examples, please refer to the *Pupillometry App* user manual (see Supplementary File1).

Pupillometry as a tool to guide electrical stimulation of the LC.

Successful targeting the LC for electrical stimulation depends on proper placement of the electrode during stereotaxic surgery. Due to its small size, consistent targeting of the LC can be challenging and incorrect placement of electrodes is common. Pupillometry readings during stereotaxic surgery can be used to immediately determine the accuracy of the placement of the implanted electrode. Acute electric stimulation during stereotaxic surgery elicits a pupil dilation when the electrode was placed in the LC (coordinates from bregma: +5.4 mm AP; +0.9 mm ML; -3.8 mm DV) but had no effect when placed 2 mm above the LC (coordinates from bregma: +5.4 mm AP; +0.9 mm ML; -1.8 mm DV, **Fig. 2c-d**). Thus, pupillometry allows the validation of correct electrode placement during surgery. Importantly, this approach might also prove useful when targeting recording electrodes to the LC, particularly when using systems that allow stimulation and recording. In addition, we performed pupillometry recordings in a two-photon setup (**Fig. 1b**) on headfixed animals with chronically implanted electrodes. Electrical LC stimulation (monophasic constant current pulse of 50 Hz, 2 ms, 200 uA for 2 s) induced pupil dilation in this setting (**Box 1**), demonstrating that the miniaturized setup can be integrated into complex experimental setups with little space.

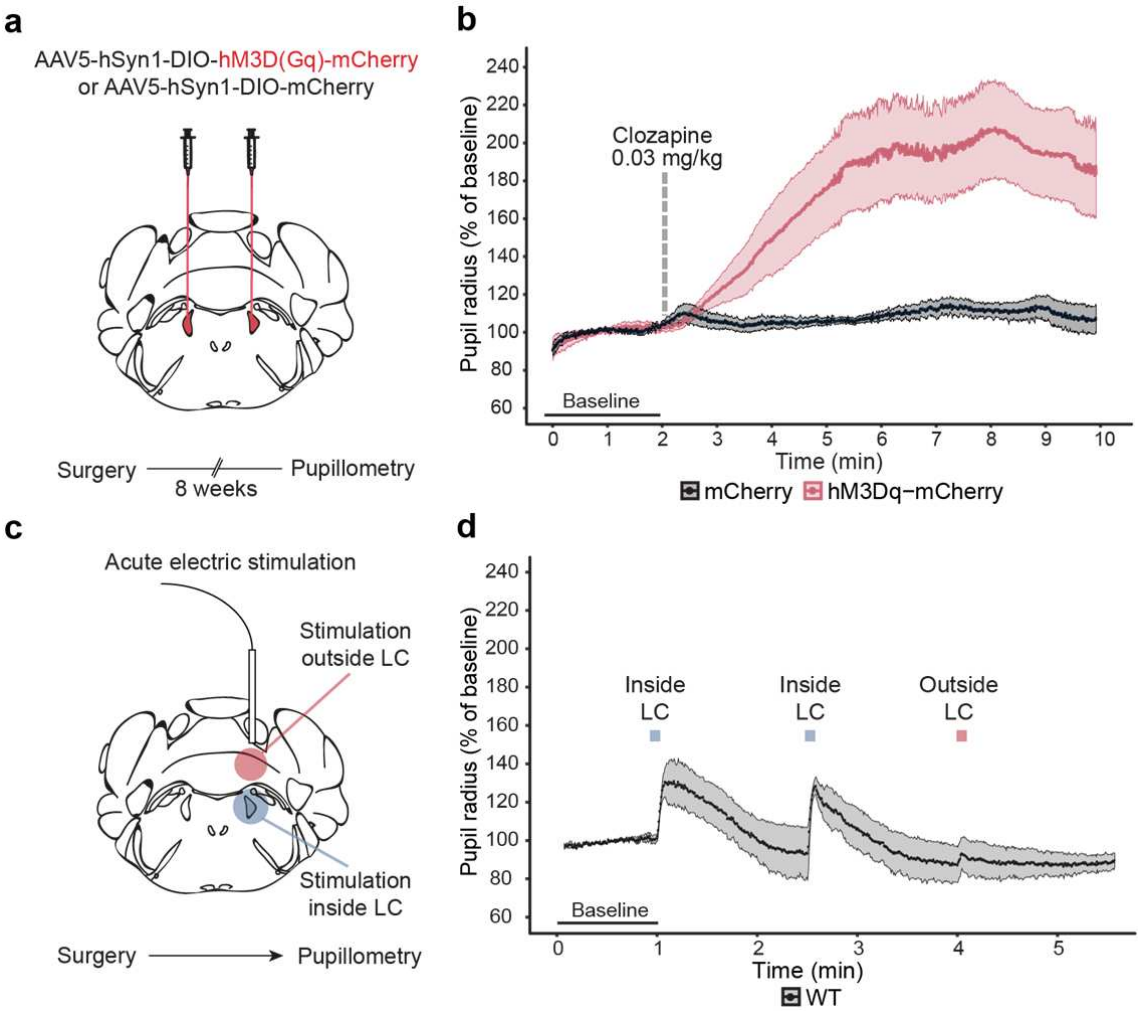


Fig. 2: Pupillometry as a real-time readout for LC activation. **(a)** Experimental design for bilateral chemogenetic LC activation. **(b)** Pupillometry traces from the right eye during chemogenetic LC activation. To see if chemogenetic LC activation induced pupil dilation in hM3Dq-mCherry (n=7) or mCherry (n=7) mice, we tested our raw data on a linear mixed effects model (Formula: *Radius ~ Stimulation * Virus + (1|Animal)*). We found a virus specific increase in pupil radius after chemogenetic LC activation (β=12.23, SE=3.930, t(28)=3.112, p=0.009). Post-Hoc analysis showed clozapine (0.03 mg/kg) injection reliably increased pupil size only in hM3Dq-mCherry (β=14.21, SE=2.78, t(12)=5.113, p= 0.0013) but not mCherry (β=1.98, SE=2.78, t(12)=0.712, p= 0.90) mice. **(c)** Experimental design for acute unilateral electric LC activation during stereotaxic surgery. **(d)** Pupil traces of wild type (WT) animals (n=3) receiving electrical stimulation inside the LC and outside the LC during stereotaxic electrode implantation. To see if electric stimulation induced pupil dilation in wild type mice (n=3), we used a linear mixed effects model (Formula: *Radius ~ Stimulation * Location + (1|Animal/Time)*). We found a significant interaction between stimulation and location (β=4.6, SE=1.53, t(7)=3.006, p= 0.019). Acute electric stimulation inside the LC elicited small yet reproducible pupil dilations (β=5.4, SE=0.88, t(7)=6.12, p= 0.0021), but had no effect on pupil size when the stimulation was given 2 mm above the LC (β=0.81, SE=1.25, t(7)=0.65, p= 0.91). All data represent mean +/- SEM. Procedures were approved under licenses ZH161/17 and ZH169/17 by the local veterinary authorities, conforming to the guidelines of the Swiss Animal Protection Law, Veterinary Office, Canton Zurich (Act of Animal Protection 16 December 2005, and Animal Protection Ordinance 23 April 2008).

Pupillometry reports physiologically relevant modes of LC activation.

The LC displays a range of firing patterns depending on the animal's behavioral state. While minimally active during sleep, LC neurons fire tonically during wakefulness at a rate that ranges from 1 Hz (drowsy/tired) up to 8 Hz under highly stressful conditions[2,4,43,44]. In

response to salient stimuli, the LC fires short high-frequency bursts (3-5 action potentials at 10-20 Hz)[43,45,46]. To demonstrate that pupillometry can discriminate between different, physiologically relevant stimulation paradigms, we combined optogenetic LC stimulations and pupil recordings in DBH-iCre mice that unilaterally expressed either channelrhodopsin (AAV5-hEF1a-DIO-hChR2(H134R)-EYFP; ChR2-EYFP) or a control virus (AAV5-hEF1a-DIO-EYFP; EGFP) in noradrenergic LC neurons (**Fig. 3a**). A short burst of optogenetic LC stimulation (15 Hz, 4 pulses, 10 ms each pulse) reliably triggers a transient pupil response, and increasing frequencies of tonic LC stimulation reveal a graded pupil response, which is absent in control mice (**Fig. 3b**). It is important to note that the higher response of tonic vs. phasic stimulation is presumably due to the higher number of stimulation pulses that were delivered over the 10 second period in the tonic paradigm, whereas the phasic stimulation was a single burst of only 4 pulses (see **Fig. 3c**). Despite the fact that the pupil is light sensitive, exposure to flashing 473 nm laser light had no effect on pupil size (EGFP control group), even under dim lighting (30 lux). Importantly, noxious stimuli can trigger LC activation under anesthesia[47,48], thus we delivered electrical shocks to the tail (biphasic constant current pulse of 30 Hz, 2 ms, 200 uA for 5 s) while recording pupil size. We observed that tailshock reliably induced pupil dilations (**Supplementary Fig. 1**). This demonstrates that physiological LC responses can also be measured using pupillometry.

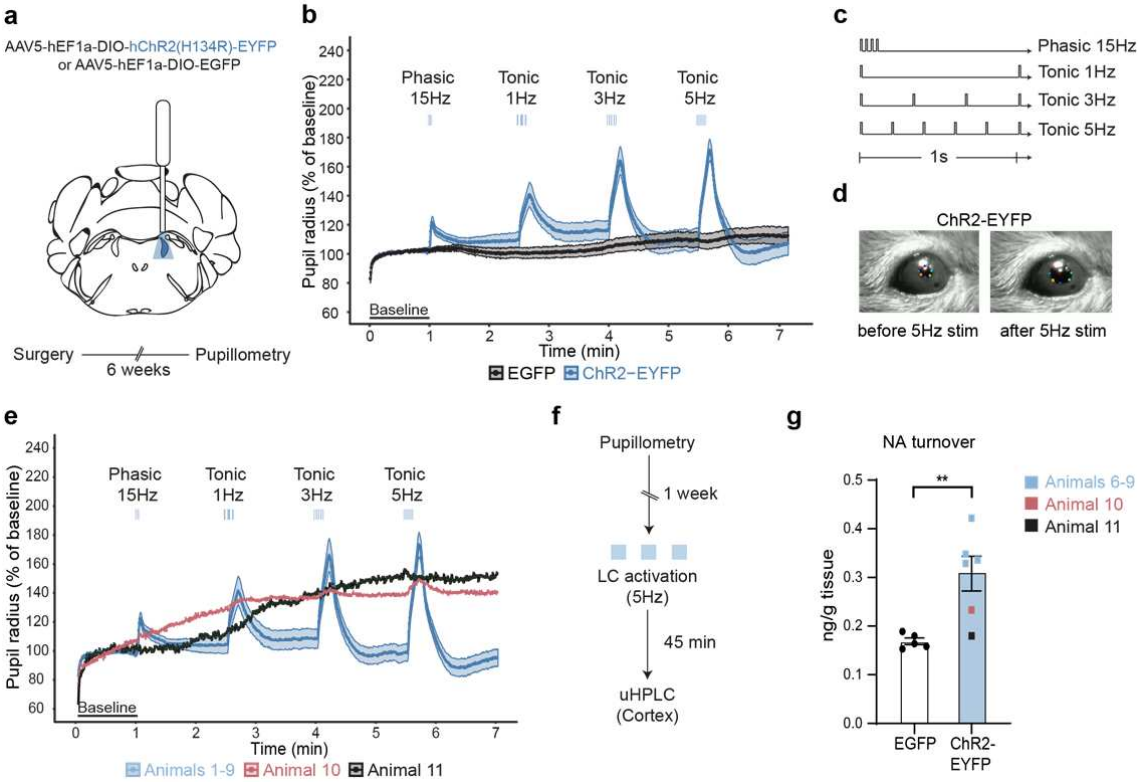


Fig. 3: Pupillometry is a sensitive readout of physiologically relevant modes of LC activity. (a) Experimental design for unilateral optogenetic LC activation. **(b)** Pupillometry traces from the ipsilateral eye. To see if optogenetic LC activation induced pupil dilation in ChR2-EYFP (n=11) or EGFP (n=10) mice, we tested our raw data on a linear mixed effects model (Formula: *Radius ~ Stimulation * Virus + (1|Animal/Time)*). We found a virus specific increase in pupil radius after optogenetic LC activation (β=5.43, SE=0.626, t(168)=8.678, p<0.0001). Post-hoc analysis showed that phasic (15 Hz, 4 pulses, 10 ms each pulse) and tonic (1 Hz/3 Hz/5 Hz for 10 s, 10ms each pulse) firing patterns reliably increased pupil size only in ChR2-EYFP (β=5.47, SE=0.432, t(82)=12.678, p<0.0001) but not EGFP (β=0.04, SE=0.453, t(82)=0.099, p= 0.99) mice. **(c)** Optogenetic

stimulation paradigms for phasic and tonic LC activation. **(d)** Images showing the pupil size before and after optogenetic LC activation with 5 Hz, while being tracked by our DLC network. **(e)** Pupil traces of ChR2-EYFP animals separated according to their response to LC activation. Optogenetic LC activation induced strong pupil dilations in 9 out of 11 animals (Animals 1-9), but in 2 animals it elicited only a very weak (Animal 10) or no response (Animal 11). **(f)** Experimental design to evaluate noradrenaline (NA) release in the brain. One week after pupillometry recordings, NA turnover (as calculated by the MHPG/NA ratio) was assessed 45 min after LC stimulation (5 Hz /10 ms pulses, alternating 3 min bins off/on for 18 min[4]) in the cortex of EGFP (n=5) and ChR2-EYFP (n=6) animals. **(g)** LC activation increased cortical NA turnover in ChR2-EYFP mice (unpaired t test; t(9) = 3.50, p = 0.0067). In accordance with pupillometry results, animal 10 showed only a weak increase, and animal 11 showed no change in cortical NA turnover. All data represent mean +/- SEM. Procedures were approved under licenses ZH161/17 and ZH169/17 by the local veterinary authorities, conforming to the guidelines of the Swiss Animal Protection Law, Veterinary Office, Canton Zurich (Act of Animal Protection 16 December 2005, and Animal Protection Ordinance 23 April 2008).

Pupillometry as a tool to guide experimental decisions.

Similar to electrical LC stimulation, optic fiber placement is crucial for successful optogenetic experiments. Compared to classic *post-mortem* validation techniques, pupillometry enables easy and reliable assessment of LC activation in each individual animal, before the start of an experiment. When performing optogenetic experiments (**Fig. 3a-c**), we noticed that two mice showed very weak (Animal 10) or no (Animal 11) pupil dilation in response to stimulation (**Fig. 3e, Supplementary Fig. 2**). To determine whether LC activation was indeed unsuccessful in these animals, we stimulated all mice optogenetically (5 Hz/10 mW) 45 min before sacrifice[4], and measured cortical NA turnover applying reversed-phase ultra-high performance liquid chromatography (uHPLC). We have shown previously that our uHPLC method[39] is a highly sensitive and reliable readout of LC activation and alteration, as previously measured both in rodent, as well as in human brain tissue and biofluids[24,49–51]. In accordance with our pupillometry data, all mice responded with a strong increase in NA turnover, except for the two mice where the pupil response was weak or absent (**Fig. 3f**). This confirms that pupillometry is a highly sensitive readout for successful LC stimulation, and, indeed, allows decisions about the inclusion/exclusion of animals to be made prior to further experimental procedures. This will further refine the use of animals in research.

Pupillometry to explore LC function.

Pupillometry also opens the possibility to address fundamental biological questions about the LC. We measured the pupil response after repeated optogenetic stimulation at different timepoints following surgery (2, 4 or 6 weeks) in male and female mice to determine i) if sex affects the pupil response, ii) if pupillometry recordings are stable over the course of several weeks, and iii) if pupillometry can be used to select an appropriate laser power for LC activation. First, the pupil response to optogenetic LC stimulation showed no differences between males and females (**Fig. 4b**). Second, the overall pupil response to optogenetic LC activation did not change over the studied time period (**Fig. 4c,d**). Third, after averaging across time and sex, we again observed that different LC stimulation patterns led to different pupillary responses (**Fig. 4e,f**). Within a recording session, repeated presentation of a phasic burst stimulus triggers a reliable increase in pupil radius. Interestingly, 5Hz stimulation with different laser intensities (5 mW vs. 10 mW) led to the same pupil response (**Fig. 4e,f**). Therefore, we also tested whether we could trigger a reliable pupil response with only 1 mW stimulation intensity. Indeed, tonic stimulation (5Hz, for 10 s, 10ms each pulse) at 1 mW also elicited a clear pupil response, which we also quantified in absolute values (millimeters) (**see Box 3** for absolute quantification of pupil size). Because exposure to strong laser light can

adversely affect neuronal function[52,53], pupillometry can be used to verify optimal stimulation paradigms and lower stimulation intensities.

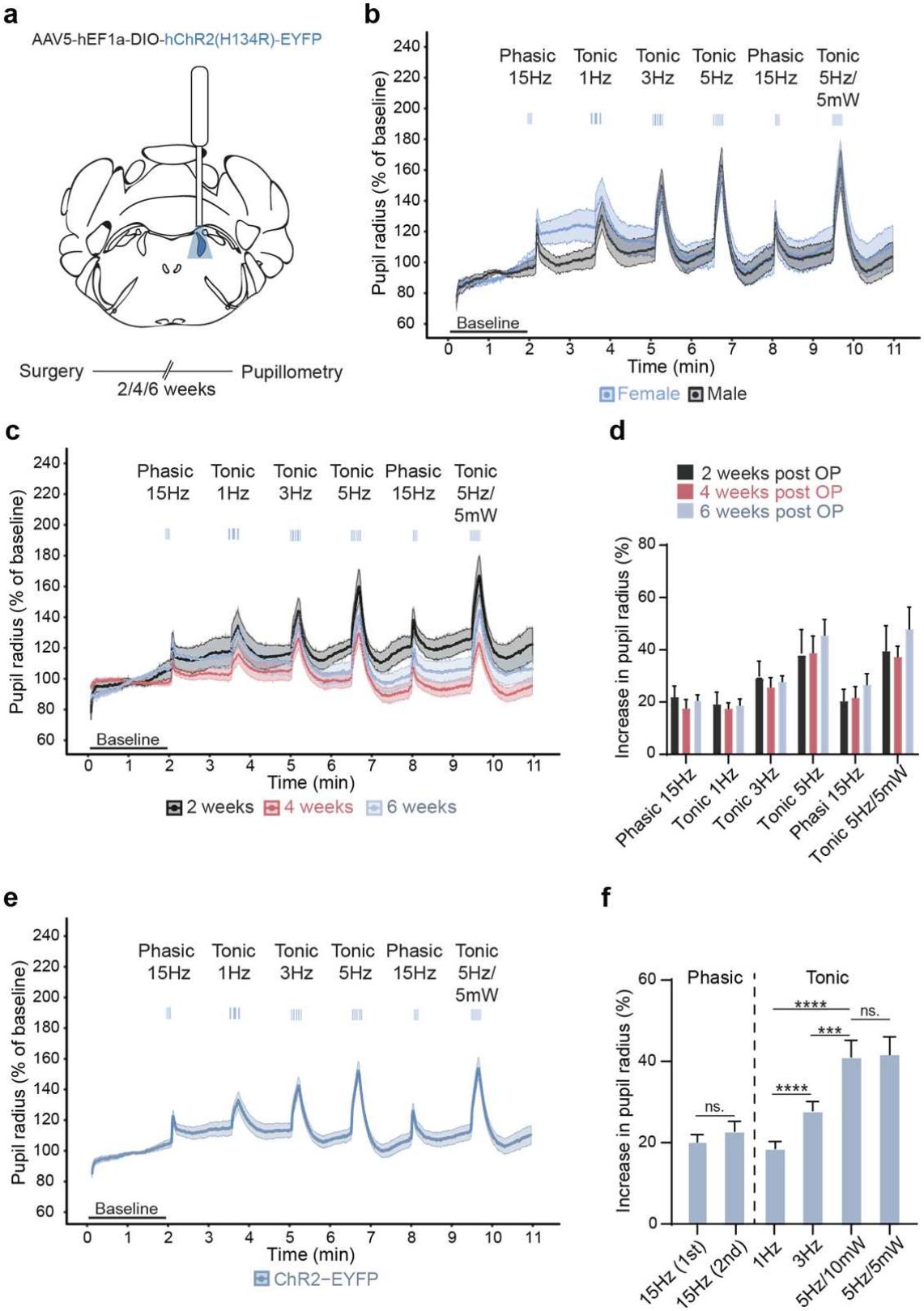
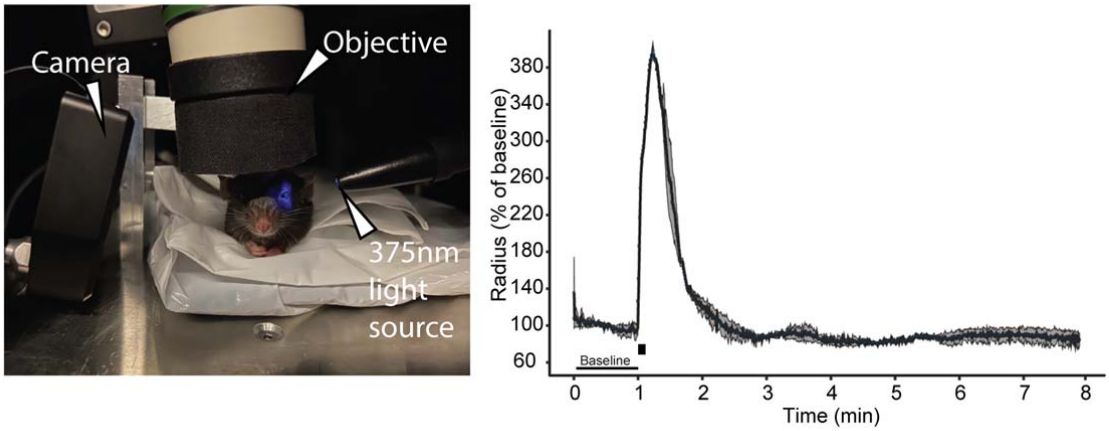


Fig. 4: Repeated pupillometry recordings with various stimulation parameters in females and males. (a) Experimental design of unilateral optogenetic LC activation. To assess effects of optogenetic stimulation at different timepoints after surgery, frequencies (as described in Figure 3c), sex, and laser power (5 Hz / 5 mW vs. 5 Hz / 10 mW) on the pupil radius. **(b)** Pupillometry traces (2, 4 and 6 weeks combined) of female (n=5) and male (n=6) ChR2-EYFP mice. To see if there is a sex-dependent difference in pupil response upon LC stimulation, we used a linear mixed effects model (Formula: *Radius ~ Stimulation* Sex + (1|Animal/Week post OP/Time)*). We see an increase of pupil size in response to optogenetic LC activation (β=3.45, SE=0.237, t(196)=14.573, p<0.0001) but did not detect any sex-related differences in pupil response (β=0.46, SE=0.320, t(396)=1.429, p=0.155). **(c)** Pupillometry traces (female and male mice combined, n=11) at 2, 4 or 6 weeks after surgery. We used a linear mixed effects model (Formula: *Radius ~ Stimulation * Week post OP + (1|Animal/Time)*) to assess if there is a difference between optogenetic LC stimulation and time after surgery. We see an increase of pupil size in response to optogenetic LC activation (β=3.75, SE= 0.960, t(396)=3.906, p=0.0001) but did not detect any time-related differences in pupil response to stimulation (Week 4; β=0.04, SE=1.357, t(380)=0.028, p=0.978; Week 6; β=0.11, SE=1.357, t(380)=0.083, p=0.934). **(d)** For each stimulation frequency we detected no time-dependent differences in pupil size increase (repeated measures two-way ANOVA; F(10, 150)= 0.5073, p= 0.88). Instead, the strength of the pupil response was mediated by the stimulation frequency (repeated measures two-way ANOVA; F(1.959, 19.59)= 23.03, p<0.0001). **(e-f)** Visualization of the stimulation dependency of LC mediated pupil responses, averaged across sexes and time points (n=11). **(f)** Repeated measures one-way ANOVA reveals that different LC stimulation paradigms differentially impact pupil dilation (F(2.128, 68.10)= 26.00, p<0.0001). Tukey post-hoc tests show no difference between a 15 Hz pulse at the beginning (1st) and towards the end (2nd) of the recording. However, increasing frequencies of tonic stimulation paradigms led to a graded increase in pupil dilation. We detect no difference in pupil response between tonic stimulation with 5 mW and 10 mW. ***=p<0.001; ****=p<0.0001. All data represent mean +/- SEM. Procedures were approved under licenses ZH161/17 and ZH169/17 by the local veterinary authorities, conforming to the guidelines of the Swiss Animal Protection Law, Veterinary Office, Canton Zurich (Act of Animal Protection 16 December 2005, and Animal Protection Ordinance 23 April 2008).

Text Boxes

Box 1| Pupillometry in darkness

Certain experimental procedures, e.g. two-photon imaging, require the pupil to be recorded in absence of (visible) light. When performing pupillometry in darkness, the pupil may already be fully dilated. To pre-constrict the pupil, a small non-dispersing light source can be pointed at the contralateral eye (not the one recorded from), to avoid additional reflections. Both pupils will then equally constrict, owing to the consensual pupillary light reflex (see Box 2). The image below shows a mouse in a two-photon imaging setup. The right eye is recorded with a Raspberry Pi NoIR V2 camera while the left eye is illuminated with UV light (375nm) to pre-constrict the pupil otherwise dilated in absolute darkness. The graph on the right shows the pupil response to electrical LC stimulation (monophasic constant current pulse of 50 Hz, 1 ms, 200 uA for 2 s) after 1 min of baseline (n=2).



Hardware & Equipment

- 375nm 5mm UV-LED (e.g. NSPU510CS)
- 68 ohm resistor (depends on the forward voltage of the LED and the source voltage)
- (Optional) 10k potentiometer to control LED brightness
- 2 Wires with female dupont connector on one end
- Small tube with 5mm inner diameter and a small opening on one side (e.g. from an old ball-point pen)
- Soldering equipment
- Superglue
- 5V DC source (e.g. pins 4 (+5V) and 6 (GND) on Raspberry Pi)

Procedure for mounting the LED-lightsource

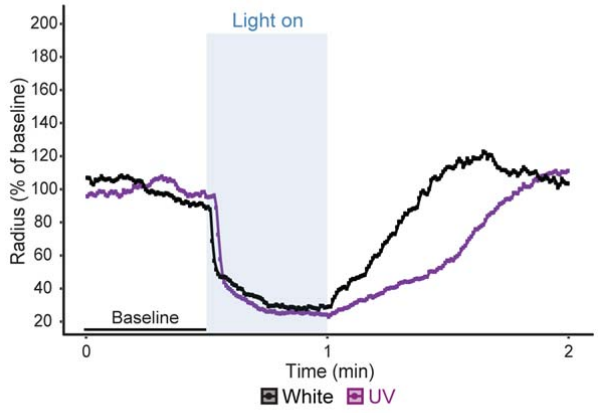
1. Solder resistor to LED cathode.
2. Solder one wire to the other end of the resistor and the other one to the LED anode. If using a potentiometer, solder one wire to one of the outer pins of the potentiometer and the other end of the resistor to the middle pin of the potentiometer.
3. Glue LED into the bigger end of the tube, pointing to the inside and wait until glue is cured.
4. Ensure correct polarity and connect the wires to 5V power.
5. Point the light at the eye contralateral to the recording

6. (Optional) Adjust light intensity by turning the potentiometer knob.

Timing
5-10 minutes

Box 2 | Recording the pupillary light reflex

The protocol presented here can be adapted with minor modifications to measure light-induced pupil diameter changes, i.e. the pupillary light reflex . The pupillary light reflex affects both eyes equally, no matter if one or both eyes are illuminated[54]. Therefore, the pupillometry recording can be performed on one eye, while the light stimuli are directed only to the contralateral eye, thus avoiding light-interference with the video recordings. We recommend using a controllable LED light source for Raspberry Pi, for example via I2C bus. By using the aforementioned light source, intensity and timing of the light stimulus may be controlled via a python script executed on the Raspberry Pi. The figure shows recording of the pupil response to a 30s stimulus (blue box) of UV (375nm; purple line) or white light (black line). The light-sources were built as described in Box 1. Traces each depict a single recording normalized to the 1 min baseline indicated at the bottom left.



Box 3| Absolute pupil measurements with DLC

The protocol presented here can be adapted to gain absolute pupil measurements. This requires a calibration object. First, the calibration object of known dimensions has to be installed in a fixed position close to the eye. It has to be oriented orthogonally to the camera and be approximately the same distance away from the camera as the eye. A new DeepLabCut network has to be trained that tracks two additional points (pc1 and pc2), one on each side of the calibration object (as shown in the figure below, panel on the left). To transform the DLC data into a metric representation, the distance between the two tracked calibration points in pixels has to first be calculated using the formula:

$$d_{px} = \sqrt{(pc1_{x\,(px)} - pc2_{x\,(px)})^2 + (pc1_{y\,(px)} - pc2_{y\,(px)})^2}$$

where pc1 is point 1 and pc2 point 2 of the calibration object and x(px)/y(px) the x/y-coordinates in pixels. We advise to use median values for pc1 and pc2 coordinates. Then,

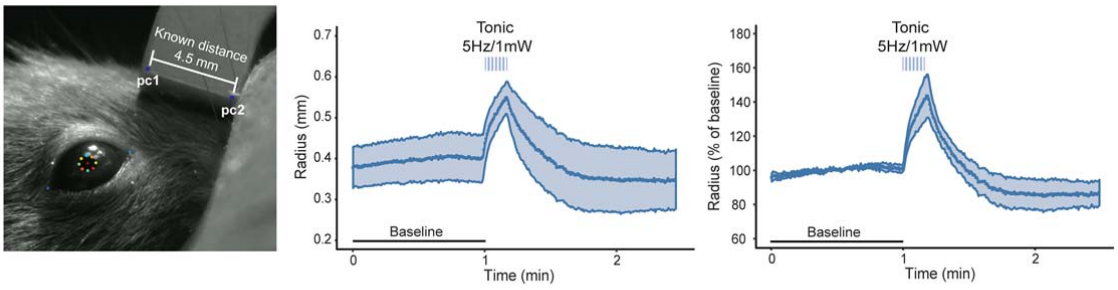
the absolute dimension of the calibration object in mm (d_{mm}) has to be divided by this value to obtain the pixel to mm conversion ratio.

$$ratio_{mm\,/\,px} = d_{mm} \,/\, d_{px}$$

Now, x and y coordinates of all tracked points at each frame can be multiplied by the conversion ratio resulting in a metric description of the tracked points.

$$p_{x\,(mm)} = p_{x\,(px)} * ratio_{mm\,/\,px}$$
$$p_{y\,(mm)} = p_{y\,(px)} * ratio_{mm\,/\,px}$$

This has to be done for each DLC ".csv" file independently and can be done with any software with relative ease (Excel, R or MATLAB). The web app is fully compatible with the transformed data, however plots will still indicate pixels in the axis description. We therefore advise using the exportable plot, which allows for custom axis descriptions. The figure shows the same pupil trace in response to optogenetic LC activation (5 Hz / 1 mW / 10 s, 10 ms pulse duration, n=4) once analyzed in absolute (millimeters, middle panel) and once in relative (% of baseline, right panel) measurements. Note that the baseline pupil size varies between mice, which leads to larger standard variation if absolute values are reported, compared to normalized measurements.



Author Contributions

Conceptualisation, M.P., K.D.F., B.W. and J.B.; Methodology, M.P., K.D.F., O.S.; Investigation, M.P., K.D.F, A.F.-S., S.N.D., Y.V., M.T.W.; Software, K.D.F., L.v.Z., P.-L.G., O.S.; Writing – Original Draft, M.P., K.D.F, L.v.Z., O.S., S.N.D. and J.B.; Figures, M.P. and K.D.F.; Writing – Review & Editing, M.P., K.D.F., L.v.Z., O.S., A.F.-S., P.-L.G., Y.V., S.N.D., M.T.W., P.P.D.D., B.W. and J.B.; Funding Acquisition, B.W., P.P.D.D., and J.B.; Resources, B.W. and J.B.

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Competing interests

The authors declare no competing interests.

Data Availability Statement

The datasets generated during the current study (Anticipated results) are available from the corresponding authors upon request.

Code Availability Statement

All software and code described in this protocol are freely available online:

1) Raspberry Pi Code
   https://github.com/ein-lab/pupillometry-raspi
2) MATLAB Code
   https://github.com/ein-lab/pupillometry-matlab
3) *Pupillometry App*
   https://bohaceklab.hest.ethz.ch/pupillometry/ (web version)
   https://github.com/ETHZ-INS/pupillometry (source code)

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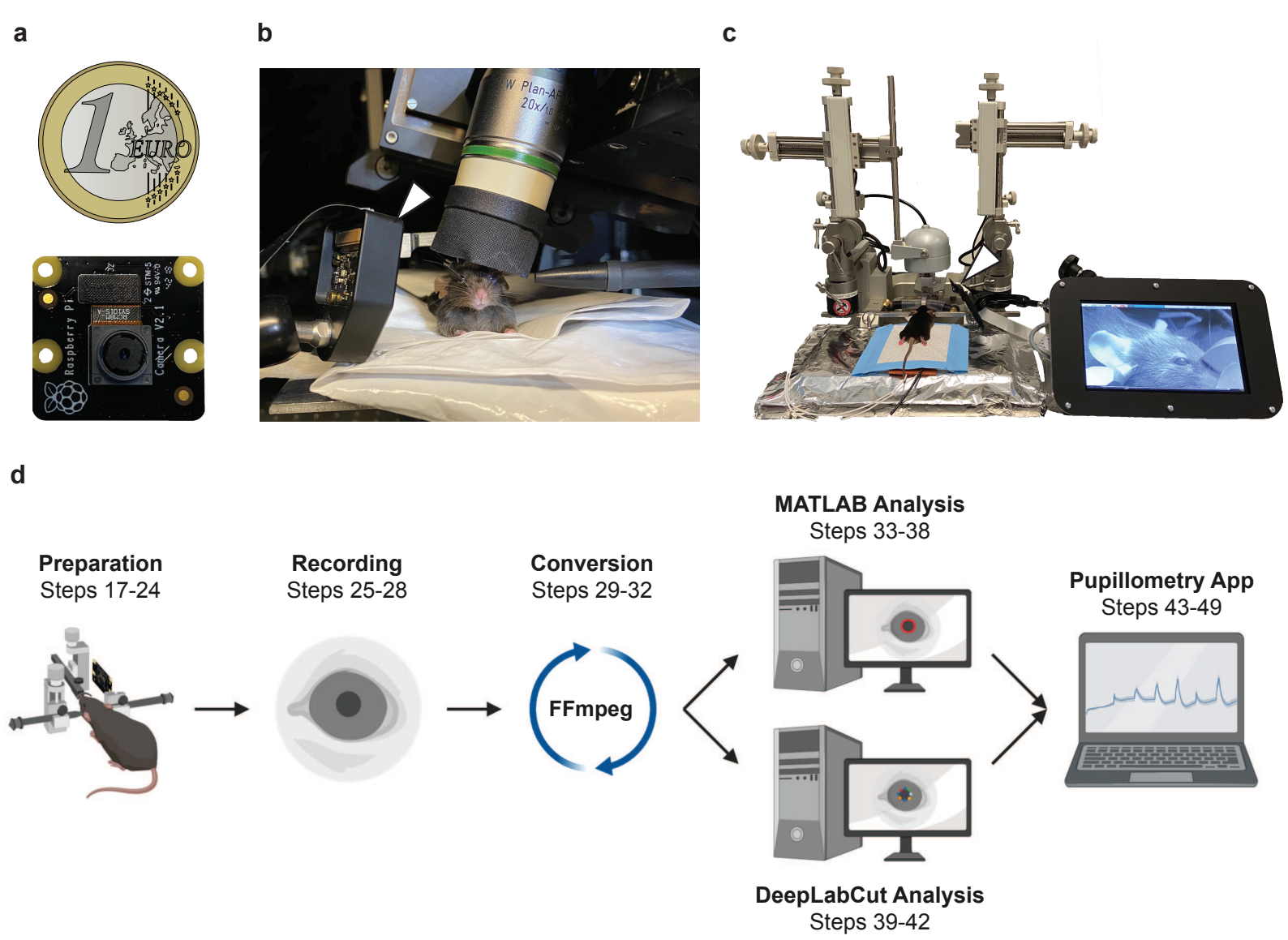
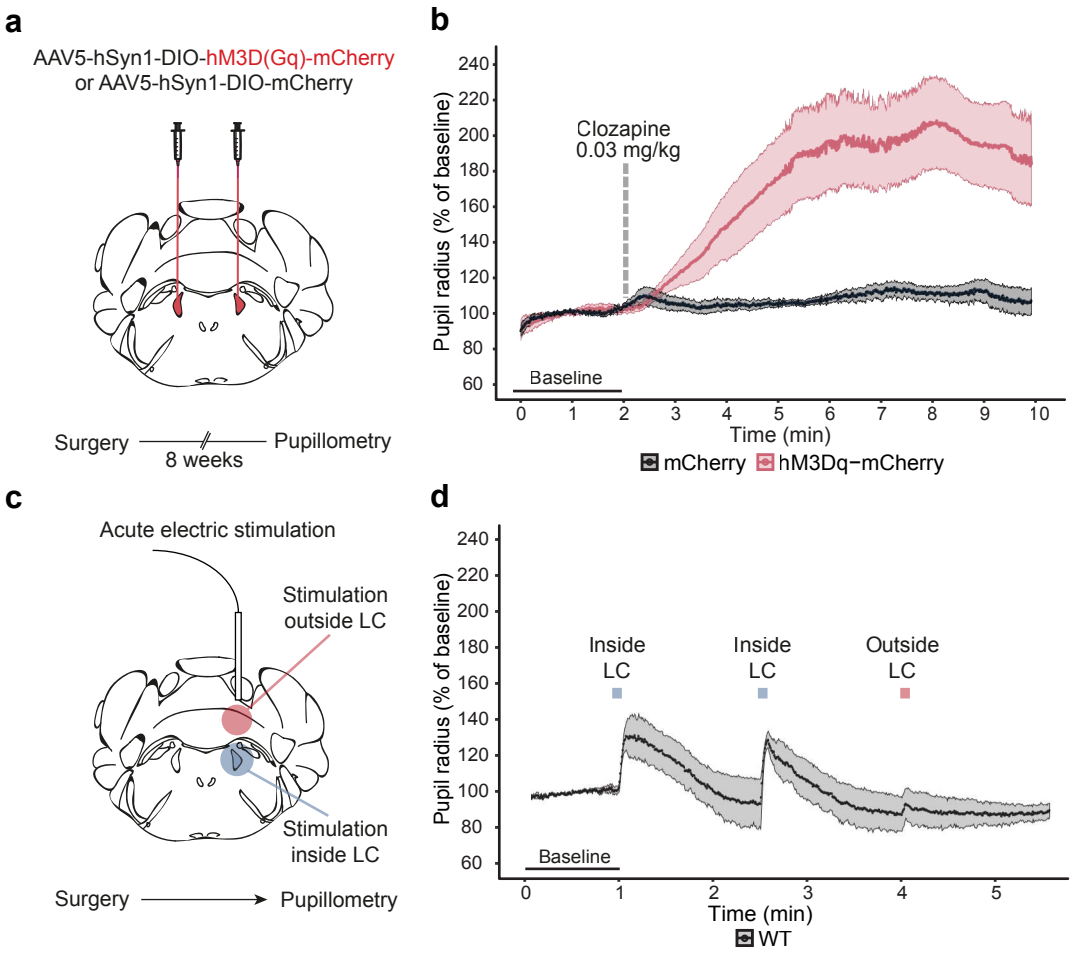
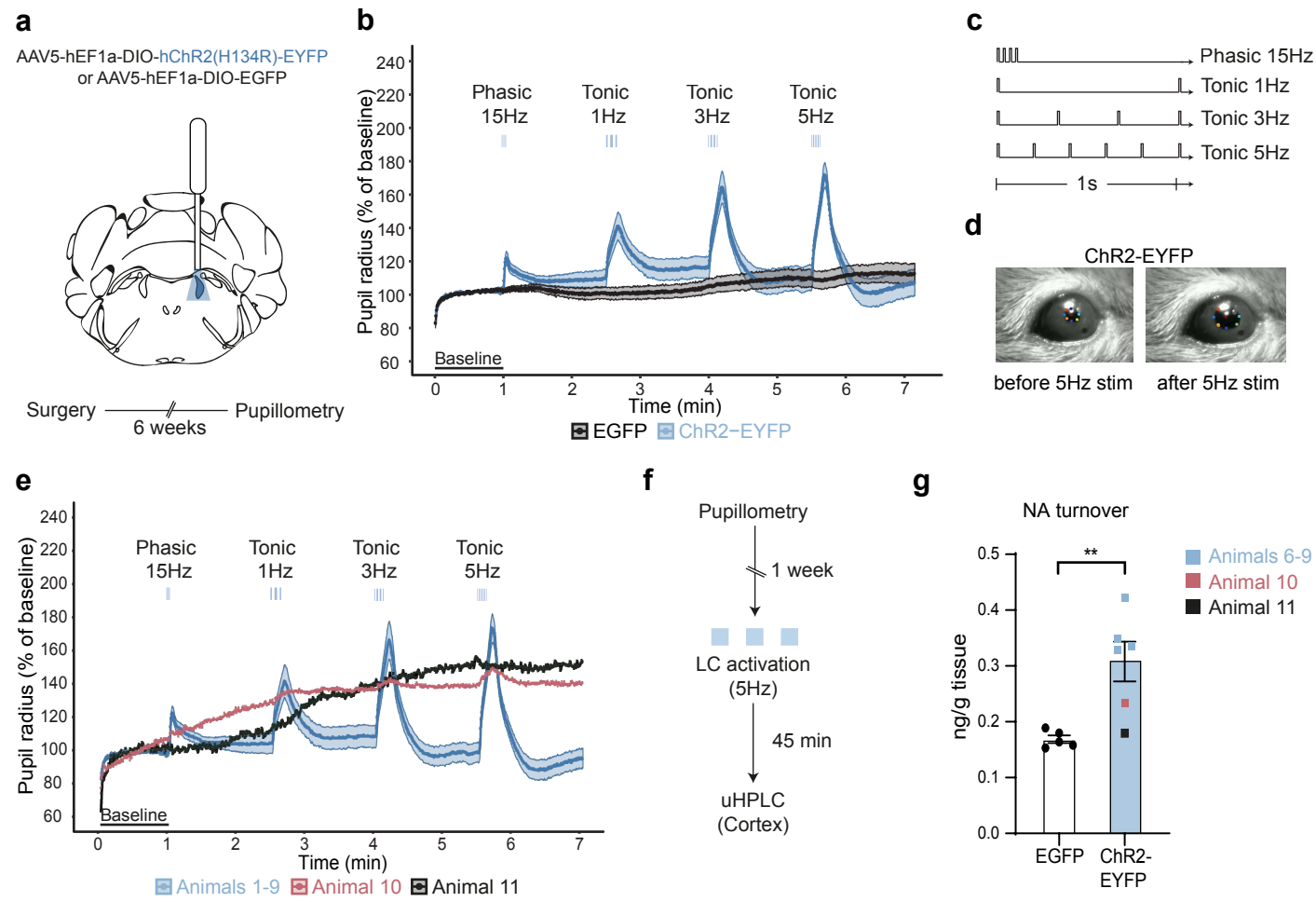
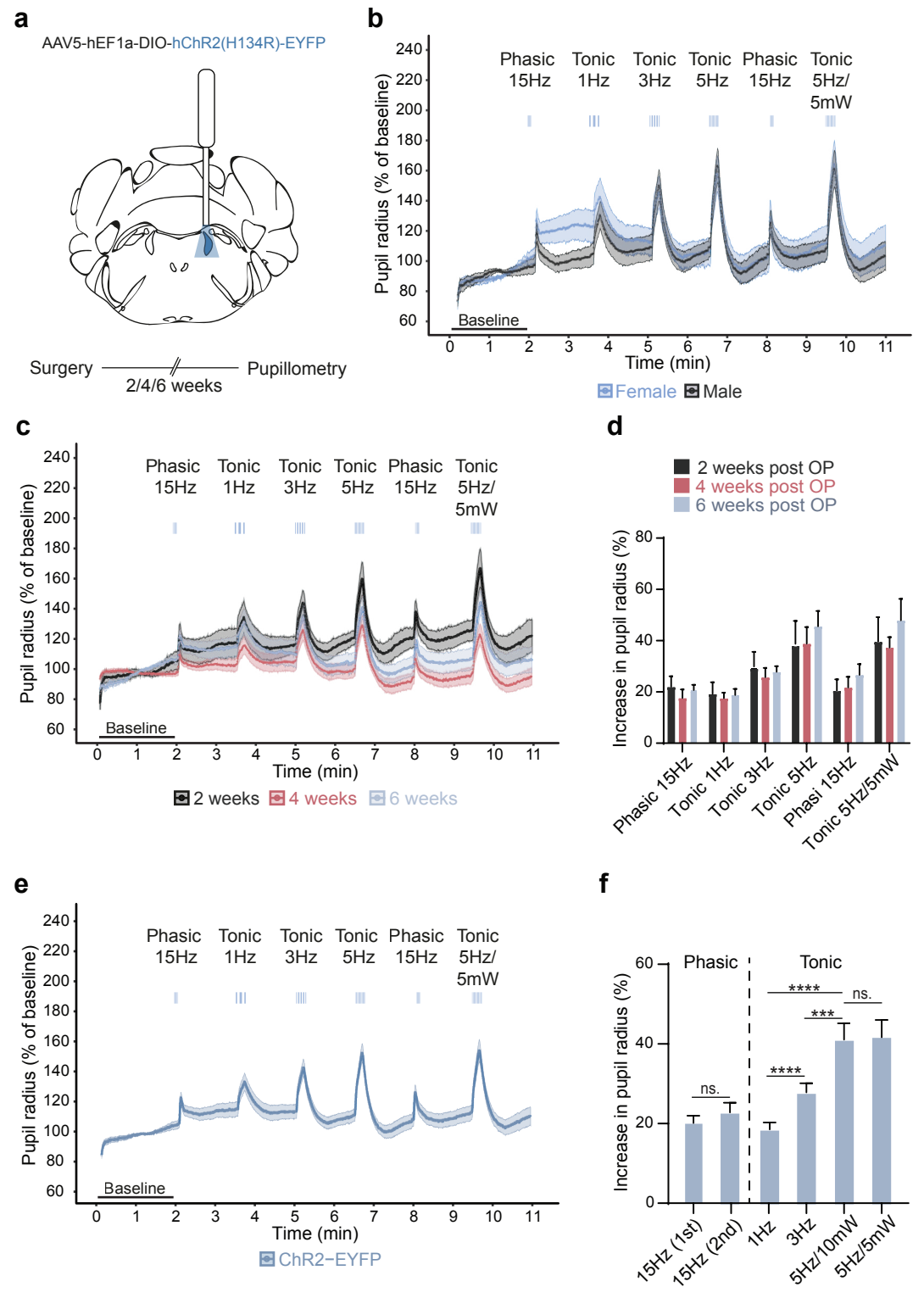
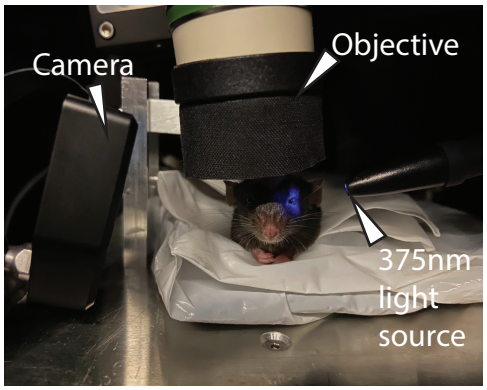

a
b
c
d
Preparation
Steps 17-24
Recording
Steps 25-28
Conversion
Steps 29-32
FFmpeg
MATLAB Analysis
Steps 33-38
DeepLabCut Analysis
Steps 39-42
Pupillometry App
Steps 43-49

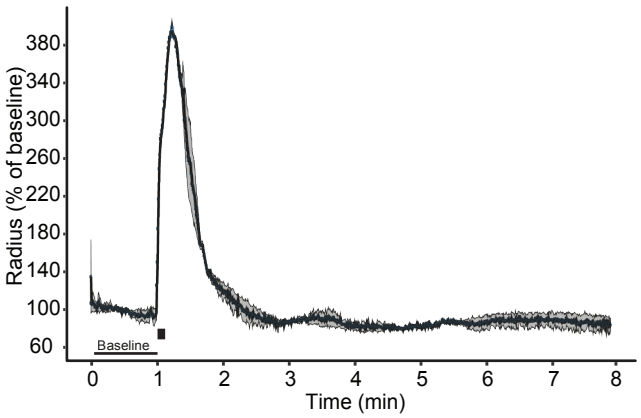

a
AAV5-hSyn1-DIO-hM3D(Gq)-mCherry
or AAV5-hSyn1-DIO-mCherry
Surgery
8 weeks
Pupillometry
b
Clozapine
0.03 mg/kg
Pupil radius (% of baseline)
Baseline
Time (min)
mCherry
hM3Dq−mCherry
c
Acute electric stimulation
Stimulation
outside LC
Stimulation
inside LC
Surgery
Pupillometry
d
Inside
LC
Inside
LC
Outside
LC
Pupil radius (% of baseline)
Baseline
Time (min)
WT

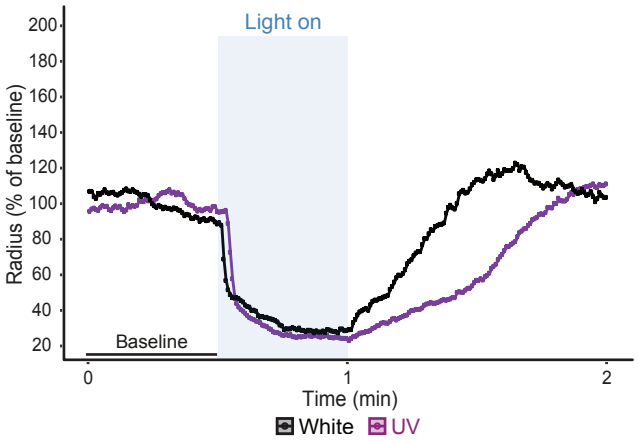

a
AAV5-hEF1a-DIO-hChR2(H134R)-EYFP
or AAV5-hEF1a-DIO-EGFP
Surgery
6 weeks
Pupillometry
b
Phasic 15Hz
Tonic 1Hz
Tonic 3Hz
Tonic 5Hz
Pupil radius (% of baseline)
Baseline
Time (min)
EGFP
ChR2-EYFP
c
Phasic 15Hz
Tonic 1Hz
Tonic 3Hz
Tonic 5Hz
1s
d
ChR2-EYFP
before 5Hz stim
after 5Hz stim

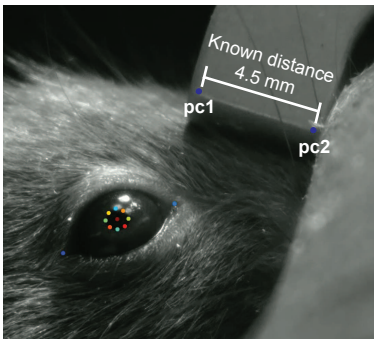
e
Phasic 15Hz
Tonic 1Hz
Tonic 3Hz
Tonic 5Hz
Pupil radius (% of baseline)
Baseline
Time (min)
Animals 1-9
Animal 10
Animal 11
f
Pupillometry
1 week
LC activation (5Hz)
45 min
uHPLC (Cortex)
g
NA turnover
**
ng/g tissue
EGFP
ChR2-EYFP
Animals 6-9
Animal 10
Animal 11

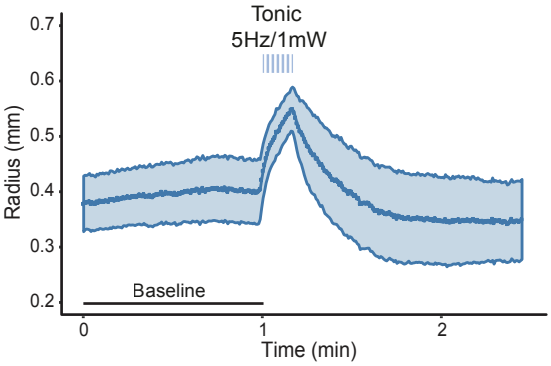

a
AAV5-hEF1a-DIO-hChR2(H134R)-EYFP
Surgery
Pupillometry
2/4/6 weeks
b
Phasic 15Hz
Tonic 1Hz
Tonic 3Hz
Tonic 5Hz
Phasic 15Hz
Tonic 5Hz/ 5mW
Pupil radius (% of baseline)
Baseline
Time (min)
Female
Male
c
Phasic 15Hz
Tonic 1Hz
Tonic 3Hz
Tonic 5Hz
Phasic 15Hz
Tonic 5Hz/ 5mW
Pupil radius (% of baseline)
Baseline
Time (min)
2 weeks
4 weeks
6 weeks
d
2 weeks post OP
4 weeks post OP
6 weeks post OP
Increase in pupil radius (%)
Phasic 15Hz
Tonic 1Hz
Tonic 3Hz
Tonic 5Hz
Phasi 15Hz
Tonic 5Hz/5mW
e
Phasic 15Hz
Tonic 1Hz
Tonic 3Hz
Tonic 5Hz
Phasic 15Hz
Tonic 5Hz/ 5mW
Pupil radius (% of baseline)
Baseline
Time (min)
ChR2-EYFP
f
Phasic
Tonic
ns.
****
***
****
ns.
Increase in pupil radius (%)
15Hz (1st)
15Hz (2nd)
1Hz
3Hz
5Hz/10mW
5Hz/5mW

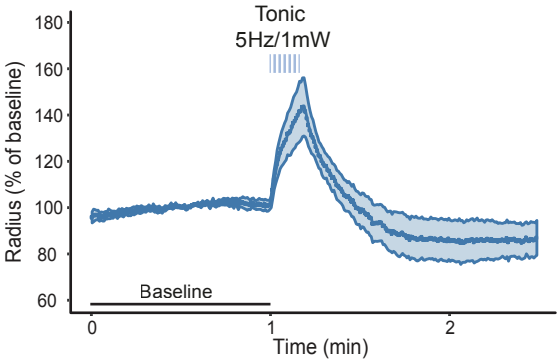

Camera
Objective
375nm
light
source


Radius (% of baseline)
380
340
300
260
220
180
140
100
60
Baseline
0
1
2
3
4
5
6
7
8
Time (min)


Light on
200
180
160
140
120
100
80
60
40
20
Radius (% of baseline)
Baseline
0
1
2
Time (min)
White
UV


Known distance
4.5 mm
pc1
pc2


Tonic
5Hz/1mW
Radius (mm)
0.7
0.6
0.5
0.4
0.3
0.2
Baseline
0
1
2
Time (min)


Tonic
5Hz/1mW
Radius (% of baseline)
180
160
140
120
100
80
60
Baseline
0
1
2
Time (min)